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Non-Selectivity of the Monoclonal Antibody M35 for Subtypes of Muscarinic Acetylcholine Receptors

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ABSTRACT: The monoclonal antibody M35, one of the first monoclonal antibodies successfully raised against muscarinic acetylcholine receptors, has been widely used to study the distribution of this protein in a variety of tissues and cell types of different species. It is not fully known, however, to which muscarinic acetylcholine receptor subtypes M35 binds. Knowledge of subtype-selectivity of M35 is a necessary step towards a functional interpretation of the obtained immunocytochemical data. The aim of the present study was to determine the subtype-selectivity of M35 employing transfected CHO-K1 cells stably expressing human m1–m5 muscarinic acetylcholine receptors separately, and to study M35 immunoreactivity in areas of rat central and peripheral tissues known to be specifically enriched in a single muscarinic acetylcholine receptor subtype. The results show that (a) all five transfected cell lines were immunopositive for M35, (b) nontransfected control cells were immunonegative, (c) the number of mAChRs expressed per cell correlated positively with the intensity of M35 immunoreactivity, and (d) cell types in aldehyde-fixed rat tissue enriched in a single m1–m4 subtypes revealed clear M35 immunoreactivity. Taken together, the present results show that M35 does not discriminate between muscarinic acetylcholine receptor subtypes. Evidently, the epitope of M35 on the receptor-protein is preserved on all muscarinic acetylcholine receptor subtypes. The epitope for M35 must, therefore, be localized on a homologous part of each subtype. © 1997 Elsevier Science Inc.

KEY WORDS: Pancreas, CHO-cells, Immunocytochemistry, Localization.

INTRODUCTION

An important step forward in the study of muscarinic acetylcholine receptor (mAChR) structure and function was the development of antibodies directed against the receptor protein. Monospecific monoclonal antibodies were obtained against membrane receptors to localize receptor proteins by immunocytochemistry [21,31]. The monoclonal antibody M35 is one of the first monoclonal antibodies successfully raised against mAChRs [2]. M35, an IgM, has several characteristic features. It cannot be used in a Sodium Dodecyl Sulphate (SDS)-PAGE Western blot, suggesting that the antigenic determinant(s) recognized by M35 are lost during the SDS electrophoresis or during the transfer to nitrocellulose [3]. M35

immunoprecipitates the active form of mAChRs [3], and partially mimics the agonist activity of acetylcholine (ACh) on guinea pig myometrium [18], but does not interfere with agonist or antagonist binding to mAChRs [40]. M35 has been used as a tool to dissect the interactions between biochemical and physiological events following mAChR stimulation, and to locate mAChRs in a variety of tissues (Table 2). Various studies describe (a) mAChR distribution at peripheral organs and cell types [5,15,18,26,30,35], (b) mAChR distribution at the cellular and subcellular level in the rat forebrain, [21,23,32,33,36,43,44], (c) the neurochemical nature of the M35-positive neurons by immunofluorescence colocalization studies [14,33,44], (d) changes during development, aging and dementia in mAChR distribution in the brain [8,34,36,40], and (e) experimentally induced plasticity in mAChR distribution [6,22,37,39].

It is not known whether M35 binds selectively to mAChR-subtypes. Previously, pharmacological and anatomical data suggested that it is unlikely that M35 binds to a single subtype of mAChRs [18,34,35], and Vasudevan and co-workers demonstrated in transfected cell lines that M35 does at least recognize the m3 subtype [41]. Knowledge of subtype selectivity of M35 is a necessary step towards a functional interpretation of the observed plasticity in M35 immunoreactivity [6,22,37,38,39]. The aim of the present study was to determine the mAChR subtype selectivity of M35 employing transfected CHO-K1 cells stably expressing human m1–m5 mAChRs separately [7,10]. After determining the subtype selectivity, cell types in rat central and peripheral tissues known to be specifically enriched in a single mAChR subtype were examined for M35 immunoreactivity.

MATERIALS AND METHODS

Transfected CHO-K1 Cells

Chinese hamster ovary cell lines (CHO-K1), stably expressing pure populations of human m1–m5 muscarinic receptors separately [8,11], were a generous gift from Dr. M. R. Brann (University of Vermont). Nontransfected CHO-K1 cells were purchased from American Type Culture Collection (Rockville, MD). Cells were grown at 37°C in 95% humidified air/5% carbon dioxide, in Minimum Essential Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% bovine serum opti-

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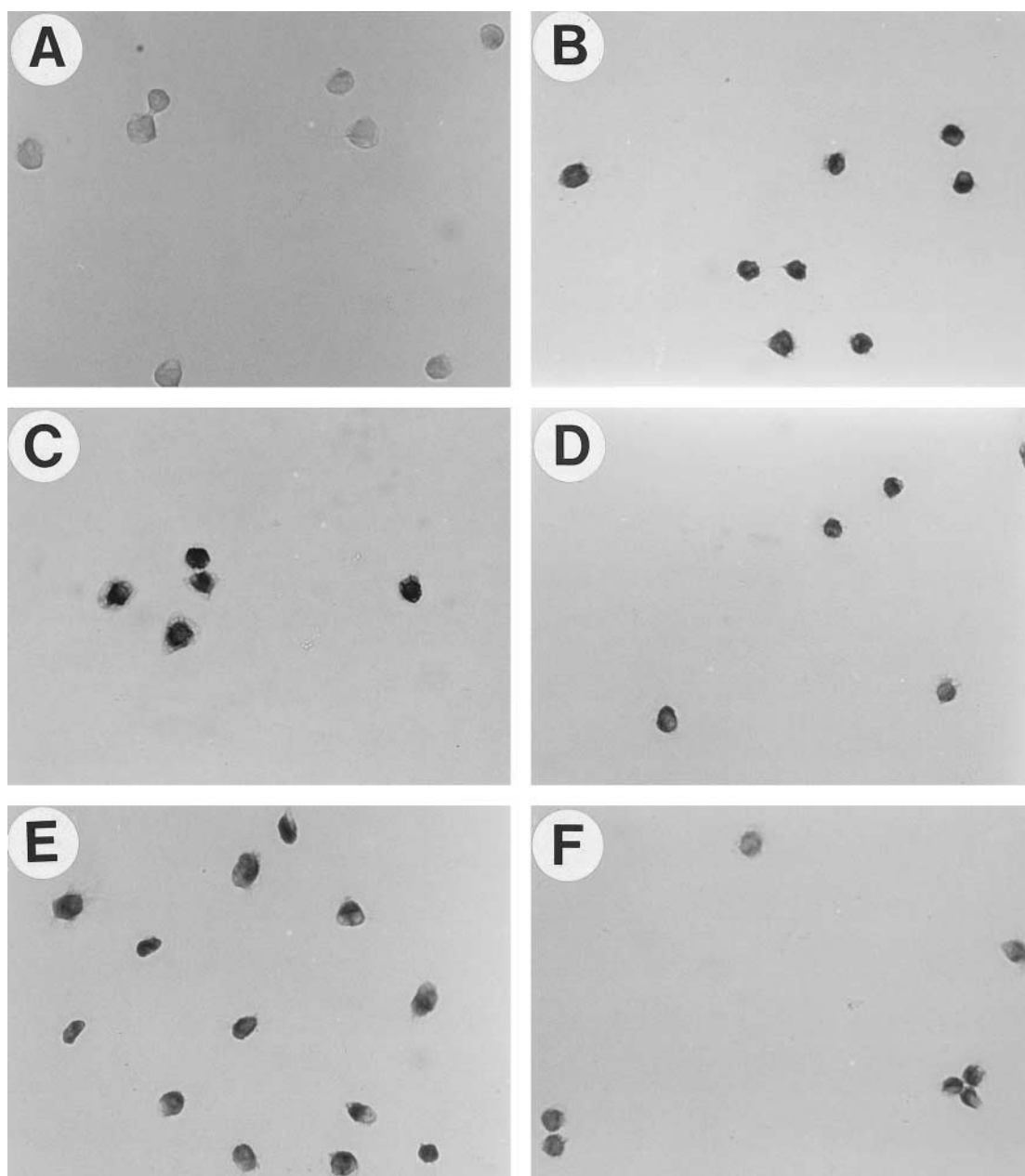


FIG. 1. M35 immunoreactivity in CHO-K1 cells expressing mAChR-subtype m1 (B), m2 (C), m3 (D), m4 (E), and m5 (F) separately. Nontransfected cells (A) were immunonegative.

mized for CHO cells (HyClone, Logan, MO), 100 units/ml penicillin, and 100 μ g/ml streptomycin. For transfected cells only, 0.1 mM gentamicin (Gibco) was included. When cells reached 80–90% confluency, they were replated on glass coverslips, coated with 100 μ g/ml poly-L-lysine (Sigma, St. Louis, MO), and allowed to attach for 2 h.

Radioligand Binding Assay

The number of mAChRs per cell was determined by radioligand binding assay. Confluent m1–m5 CHO cells were scraped from culture flasks, homogenized, and sedimented. Pellets were resuspended in 50 mM sodium phosphate buffer (PBS) (pH 7.4), con-

taining 1 mM EDTA. Aliquots of membrane homogenates were incubated in 1 ml of 0.1 nM 3 H-N-methylscopolamine (3 H-NMS) (84 Ci/mmol, Dupont-NEN, MA) for 2 h at 25°C. Membranes were collected by filtration and radioactivity was counted as previously described [29]. Nonspecific binding was assessed in the presence of 1 μ M quinuclidinyl benzilate. Specific 3 H-NMS binding to mAChRs was calculated as the difference between the total binding (absence of unlabeled ligand) and nonspecific binding (excess unlabeled ligand).

Immunocytochemical Procedure

Six adult Wistar rats (weighing 300 g) were used to study M35 immunoreactivity in the brain and pancreas. The animals

were deeply anesthetized with 6% sodium pentobarbital and perfused transcardially with 30 ml heparinized saline (15 ml/min) followed by 200 ml of a fixative solution composed of 2% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4 (PB). The brain and pancreas were removed and cryoprotected by overnight storage in 30% sucrose in PB. Thereafter, immunostaining was carried out on free-floating frozen brain sections cut coronally and frozen sections taken from the tail of the pancreas thaw-mounted on gelatin-coated slides. All sections were 20 microns in thickness.

The tissue sections were preincubated for 15 min in 0.1% H₂O₂ in 0.01 M PBS, rinsed in PBS, and immersed in 5% normal rabbit serum (NRS) in PBS for 30 min to reduce nonspecific binding in the following incubation step. The sections were then incubated with the primary antibody. M35, a monoclonal mouse anti-mAChR IgM raised against purified bovine mAChR-protein [2,3], was a generous gift from Dr. A. D. Strosberg (Institut Cochin de Genetique Moléculaire, France). M35 was diluted 1:5 in 2% NRS in PBS overnight at 4°C under gentle movement of the incubation medium. After the primary incubation, sections were rinsed in PBS and again preincubated with 5% NRS for 30 min. They were then incubated with biotinylated rabbit IgG anti-mouse-IgM (mu-chain directed, F(ab') fraction, Zymed), diluted 1:200 in PBS in 2% NRS for 2 h at room temperature (RT). Thereafter, the sections were thoroughly rinsed in PBS and incubated in streptavidin-HRP (Zymed) diluted 1:200 in PBS for 2 h at RT.

CHO-K1 cells were fixed with 4% paraformaldehyde for 10 min at RT and excess fixative was rinsed off with PBS. They were then preincubated with 10% NRS in PBS for 1 h at RT to block nonspecific antibody binding. Excess blocking medium was removed, and the primary antibody M35 was introduced at a dilution of 1:5 in 2% NRS overnight at 4°C. Cells were rinsed with PBS and incubated with biotinylated rabbit IgG antimouse IgM (mu chain directed, F(ab') fraction, Zymed) diluted 1:200 in 2% NRS for 2 h at RT. After several rinses with PBS, endogenous peroxidase activity was quenched with 0.3% H₂O₂ in methanol for 30 min. Cells were rinsed with PBS and incubated with Vectastain *Elite* ABC reagent (Vector, Burlingame, CA) for 30 min at RT. The ABC reagent is a preformed avidin-biotinylated horseradish peroxidase complex that enhances the sensitivity of this detection method.

Finally, sections and cells were processed by the 3-3 diaminobenzidine (DAB)-H₂O₂ reaction, guided by a visual control. Once the desired staining intensity was obtained, the DAB re-

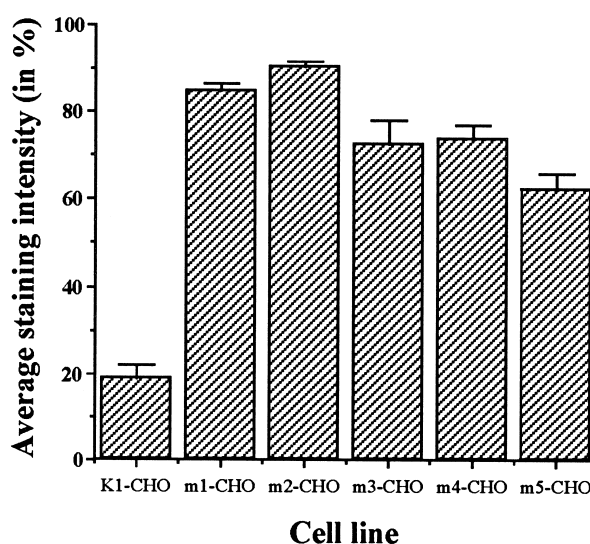


FIG. 2. Optical density measures of the M35 immunoreactivity in the transfected cell lines expressing the mAChR subtypes m1-m5 separately, as well as the control cell line K1-CHO lacking mAChRs. The optical density is expressed in averaged percent intensity (\pm SEM) of the analyzed cells as seen in Fig. 1, with 0 and 100% corresponding to maximal levels of white and black, respectively.

action was stopped by rinsing the brain sections in Tris HCl buffer, and incubating the CHO-K1 cells with deionized water for 5 min. Control experiments were performed by omitting the primary antibody (M35) from the incubation medium for rat brain sections and CHO-K1 cells. Coverslips were mounted on glass microscope slides and the staining was visualized by light microscopy.

The staining intensity of M35-immunoreactivity was measured with the aid of a scanner (SigmaScan Pro image analyzer with software by Jandel Scientific). Individual cells were scanned, and the average of the staining intensity of the cells per cell line was calculated and expressed in percentage, with 0 and 100% corresponding to minimal and maximal staining possible, respectively.

RESULTS

Binding of M35 to mAChR subtypes in transfected CHO-K1 cells

The averaged number of mAChRs expressed per cell (Table 1) varied slightly among the different cell lines, ranging from 40,813 (\pm 601) to 57,426 (\pm 445). Subsequent immunostaining with the monoclonal antibody M35 revealed that CHO-K1 cells expressing m1 (Fig. 1B), m2 (Fig. 1C), m3 (Fig. 1D), m4 (Fig. 1E), and m5 (Fig. 1F) were all immunopositive for M35, whereas nontransfected cells remained immunonegative (Fig. 1A). Likewise, omitting the primary antibody M35 from the incubation medium yielded immunonegative results (data not shown). The immunoprecipitate was present predominantly in the cell cytoplasm. No gross differences were observed among cells with different mAChR-subtypes; all cell populations were darkly stained. The optical density measures of the M35 immunostaining for the different transfected cell lines (Fig. 2) showed that the staining of the cells was approximately four times as high as in the control cell line, and that the staining intensity ranked as follows: m2 > m1 > m3 = m4 > m5. This correlated

TABLE 1

NUMBER OF mAChRs EXPRESSED IN THE FIVE TRANSFECTED CELL LINES

Cell Line	Number mAChRs per Cell (Attomoles \pm SEM)
m1-CHO	54,413 (\pm 687)
m2-CHO	57,426 (\pm 445)
m3-CHO	47,893 (\pm 566)
m4-CHO	48,240 (\pm 320)
m5-CHO	40,813 (\pm 601)

Quantitation of the number of mAChRs present per cell for the different m1-m5 CHO cell lines expressing the respective mAChR subtype separately.

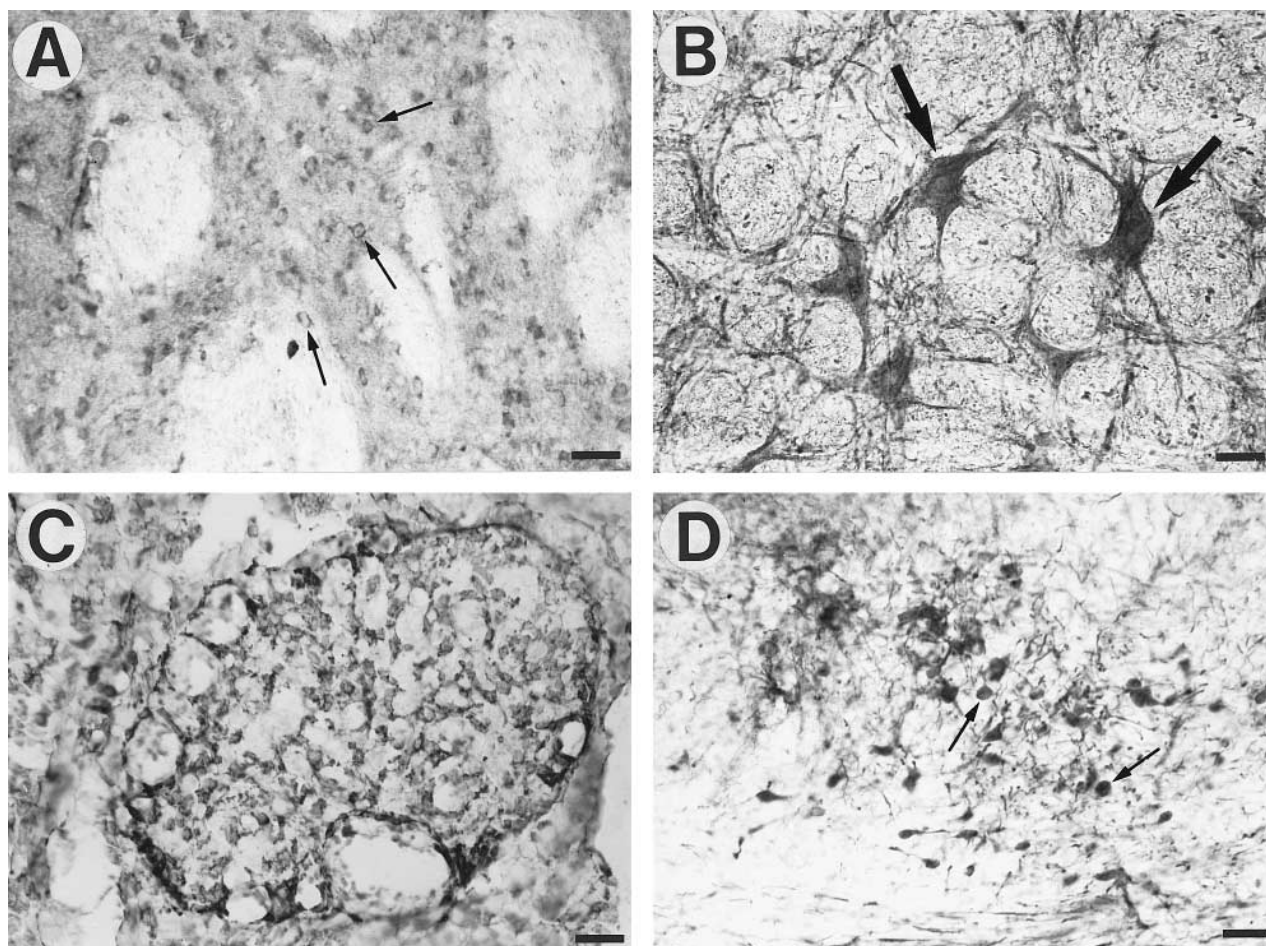


FIG. 3. M35 immunoreactivity was found in regions known to be specifically enriched in the m1 (A), m2 (B), m3 (C) or m4 (D) mAChR subtype. The striatum (A) contains numerous medium spiny cells (small arrows) which are moderately to strongly immunoreactive for M35. Motorneurons in the mesencephalon (B) are heavily labeled with M35. Islets of Langerhans in the pancreas (C) contain densely stained endocrine cells. Islands of Calleja (D) are characterized by small clusters of densely stained M35-positive neurons (small arrows). Scale bar in A, B, C, and D = 50, 20, 25, and 45 μ m, respectively.

positively and significantly with the number of mAChRs expressed per cell line ($r^2 = 0.998$; $p = 0.0012$), suggesting that the staining intensity in transfected cells relates to the number of mAChRs expressed, with no difference between the five subtypes.

M35 Binding in Rat Cells Enriched in Specific mAChR Subtypes

Cells in three brain regions and the pancreas were chosen to study M35 binding to mAChR subtypes *in situ*. Medium spiny cells in the striatum, motorneurons of the mesencephalon, endocrine cells of the islets of Langerhans in the pancreas, and cells in the islands of Calleja are selectively enriched in m1, m2, m3, and m4 mAChR subtypes, respectively [12,13,20,42]. M35 immunoreactivity was observed in all four areas (Fig. 3). The striatum contains numerous medium spiny cells (Fig. 3A), which were moderately to strongly immunoreactive for M35. Besides these neurons, large and intensely stained cells were observed mainly in the ventrolateral aspect of the striatum. The motorneurons in the mesencephalon (Fig. 3B) were heavily stained with M35, and showed characteristic pockets of dense immunoprecip-

itate around the nucleus. Immunopositive dendritic branches were frequently encountered among the motorneurons. The islets of Langerhans in the pancreas contained a mixture of lightly and densely stained endocrine cells (Fig. 3C). The densely stained cells predominated at the periphery of the islets, whereas the lightly stained cells were found in the core of the islets. Strongly immunopositive fiber-like elements were occasionally observed in the mantle of the islets. Islands of Calleja revealed small clusters of darkly stained, multipolar M35-positive neurons (Fig. 3D). Like the motorneurons in the mesencephalon, these cells have numerous immunopositive dendrites scattered throughout the island. Qualitatively similar results were obtained using 4% paraformaldehyde (data not shown) as used for the cell lines instead of 2% paraformaldehyde + 0.05% glutaraldehyde as a fixative. However, the latter fixative provides a somewhat better signal-to-noise ratio of M35 immunoreactivity.

DISCUSSION

Comparison of M35 with other mAChR Antibodies

Recent immunological localization studies for the mAChR subtypes m1 to m4 in rat brain have revealed that most brain

TABLE 2
SUMMARY OF M35-BINDING TO mAChRs IN DIFFERENT SPECIES AND TISSUES*

Species	Tissue	Reference
cat	brain	[40]
chick	brain	E. A. Van der Zee, unpublished observations
ferret	retina	[15]
frog	pars intermedia	[16]
guinea pig	brain	T. Matsuyama, unpublished observations
	myometrium	[18]
hamster	brain	E. A. Van der Zee, unpublished observations
human	brain	[24, 32]
	fibroblasts	[30]
insect	brain	E. A. Van der Zee, unpublished observations
mouse	brain	[39]
	embryo	[17]
pig	brain	[9]
rabbit	brain	[9, 38]
	smooth muscle	[26]
rat	brain	[1, 23, 25, 33, 43, 44, 45]
	heart	E. A. Van der Zee, unpublished observations
	myometrium	[18]
	pancreas	[35]
sheep	brain	B. R. K. Douma, unpublished observations
vole	brain	E. A. Van der Zee, unpublished observations

* In addition, mAChRs were detected using M35 in sperms of a large variety of invertebrate and vertebrate species by Baccetti et al. [5].

regions contain a complex mixture of mAChRs [13,19,20,27]. All cell types immunoreactive for M35 in rat brain tissue were found to express mAChRs according to some recent studies employing mAChR subtype-selective antibodies [11,19,20,27]. However, some brain regions that are not or only weakly immunopositive for M35 were found to contain mAChR immunoreactive cells in other studies. This discrepancy may be related to the occurrence of false negatives for M35. Experimental data suggest that, due to the binding characteristics of M35, immunostained brain tissue may contain some false negative muscarinic cholinceptive neurons. For example, a significant increase in the number of M35-positive neurons as well as a clear increase in dendritic labeling were found after the application of 100 μM carbachol for 15–20 min to fresh cortical brain slices. These results suggest that some of the M35 immunonegative neurons can be visualized by M35 after the internalization of the previously nondetectable mAChRs embedded within the cell membrane. Possible false negatives were found by others in the facial motoneurons and cholinergic facial nerve and olivocochlear bundle [45]. The possibility of false positives with M35 cannot entirely be excluded either. In a limited number of cases, brain regions reveal high levels of M35 immunoreactivity (notably in the central nucleus of the amygdala [33], and the cochlear root neurons [45]), while receiving only minor cholinergic innervation. Whether this strong immunostaining is due to cross-reactivity, or reflects a certain physiological state of the mAChR protein by which the epitope for M35 is maximally exposed, remains to be determined.

Epitope Localization of M35 on the mAChR Protein

This study demonstrates that M35 recognizes all five mAChR subtypes, and it is highly likely that these subtypes are also recognized in situ in aldehyde-fixed tissue. Evidently, the epitope

for M35 is preserved on all mAChR subtypes, as well as on the embryonic mAChR type [17], which is biochemically different from the adult types of mAChRs [28]. The epitope for M35 must, therefore, be localized on a part of these receptors that is highly homologous. The exact epitope recognized by M35 is unknown. However, biochemical data have revealed that M35 most likely recognizes a conformational determinant only present on the “active” receptor (i.e., the nondenatured, intact mAChR protein [3,4]). The observation that M35 can mimic some physiological effects of ACh [18] suggests that it binds near or at the ACh binding site. However, occupation of the agonist and antagonist binding sites on the mAChRs by specific ligands could not block M35 binding [40], indicating that the binding site for M35 is not identical to the ACh binding position.

M35 binding to mAChRs has been studied in a variety of species, tissues, and cell types (Table 2), and to our knowledge no species are reported in which M35 is incapable of binding to mAChRs. This strongly suggests that the M35 epitope is preserved among mAChR subtypes as well as among species, and the epitope might represent a crucial part of the functional mAChR protein.

In summary, the present results show that all five mAChR subtypes (m1–m5) are recognized by M35 in transfected cells and that at least m1–m4 are recognized in situ in rat brain or pancreas. However, due to the immunological characteristics of M35 and the location of its epitope on the mAChR protein, binding of M35 to mAChRs is determined by the physiological (functional) state of the protein. In conclusion, the monoclonal antibody M35 is a very useful tool to study plastic changes in mAChRs characteristics, whereas antibodies raised against mAChR peptide reveal a more static pattern of mAChR expression, less sensitive to the prior (behavioral) history of the animal. Combined approaches to study mAChR characteristics with M35 and subtype-specific antibodies, therefore, will provide better in-

sight in the regulation of cholinergic neurotransmission at the level of mAChRs.

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